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(57) Abstract

A method for evaluating the ability of a treatment to adversely affect the growth or viability of a cell is described. A cell having reduced susceptibility to apoptosis is provided, a treatment is administered to this cell, and a determination is made as to whether the treament affects the value of a parameter related to the growth or viability of the cell. Also described are diagnostic and anticancer therapies. Cells and cell lines used in these methods are also provided.

ANTICANCER AGENTS AND APOPTOSIS

This application is a continuation-in-part application of pending application Serial No. 08/110,127, filed on August 20, 1993, and entitled ANTICANCER AGENTS AND APOPTOSIS. The entire contents of the parent application are hereby expressly incorporated by reference.

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of Grant Nos. R01CA40602 and 5R27CA17575 awarded by the National Cancer Institute, and Grant Nos. P01-CA42063 and P30-CA14051 awarded by the National Institutes of Health.

Field of the Invention

This invention relates generally to cancer therapies, e.g, to screens for identifying anticancer agents and to cells for use in such screens.

Background of the Invention

The identification of effective anticancer agents has long been an objective in medicine. Both radiation-based therapy and chemotherapy have had a significant impact on the treatment of cancer. Major impedients to successful therapy, however, include the failure of some tumor types to respond to either form of treatment, and the appearance of resistant cell populations upon relapse of an originally responsive malignancy. Consequently, the underlying basis of cellular resistance to anticancer agents has been the focus of much experimental study. In general, these investigations have examined how chemotherapeutic agents reach their intracellular targets or the molecular nature of the drug-target interaction. The mechanisms by which anticancer agents cause cell death, however, have thus far remained elusive.

and a determination is made as to whether the treatment affects the value of a parameter related to the growth or viability of the cell.

The method provides for a first condition which can include a mutation in a gene that affects the apoptotic pathway, e.g., in a tumor suppressor gene, e.g., the tumor suppressor p53 gene. The mutation can be homozygous or heterozygous. In preferred embodiments, the method also includes a second condition which in a wild type background enhances the susceptibility of the cell to apoptosis. The second condition includes, e.g., an expressed oncogene, e.g., the adenovirus E1A gene or the c-myc gene. In more preferred embodiments, the method also includes a third condition which allows establishment of a permanent cell line when the second condition is present and is an expressed oncogene. The third condition includes, e.g., an expressed oncogene, e.g., T24 H-ras.

In one embodiment of the method of the invention, a second treatment, e.g., a chemotherapeutic agent or radiation is administered to the cell, which in the presence of tumor suppressor gene p53 activity would reduce the growth or viability of the cell. This embodiment allows identification of agents that bypass, restore or replace apoptotic pathway functions.

Variations of this method of this invention include the cell being obtained from a cell culture, e.g., a mouse embryo fibroblast cell culture, or the cell being part of an organism, e.g., an animal, e.g. a transgenic animal, that is wild type or homozygous or heterozygous for a mutation in the apoptotic pathway.

Another aspect of the invention features identifying an agent useful for treating unwanted cell proliferation by providing a cell with a first condition which reduces susceptibility of the cell to apoptosis and a second condition which in a wild type background enhances susceptibility of the cell to apoptosis. An agent is

tumor suppressor gene p53 mutation. If a p53 mutation is absent, then a therapeutically effective amount of a treatment is administered, the treatment being a p53-dependent treatment, or a combination of a p53-dependent and a p53-independent treatment. If a p53 mutation is present, then a therapeutically effective amount of a treatment is administered, the treatment being a p53-independent treatment, or a sufficiently high level of a normally p53-dependent treatment so as to overcome the p53-dependent property of the p53-dependent treatment.

And yet another aspect of the invention includes a method of treating a cell which is tumorigenic by administering a therapeutically effective amount of tumor suppressor gene p53 protein or DNA in an amount sufficient to give expression of a therapeutically effective amount of p53 protein. Preferably, the cell is deficient in tumor suppressor gene p53 function. In one embodiment, the cell is part of an organism. In another embodiment, the cell is obtained from a tumorigenic cell culture, and additionally, a therapeutically effective amount of treatment with a chemotherapeutic agent or radiation is administered.

In addition, mouse embryonic fibroblast cells that are either homozygous or heterozygous for a tumor suppressor gene p53 mutation and have an adenovirus E1A gene and a T24 H-ras gene are provided. Mouse embryonic fibroblast cell lines that are either homozygous or heterozygous for a tumor suppressor gene p53 mutation and have an adenovirus E1A gene and T24 H-ras gene are also provided.

It is an object of the invention to evaluate the ability of a treatment to adversely affect the growth or viability of a cell.

It is another object of the invention to identify anticancer agents.

It is yet another object of the invention to identify anticancer agents that are effective for cancers that are not responsive to currently available chemotherapeutic agents or radiation therapy.

Still another object of the invention is to treat an organism with a tumor by administering tumor suppressor gene p53 protein or DNA.

The above and other features, objects and advantages of the present invention will be better understood by a reading of the following specification in conjunction with the drawings.

Brief Description of the Drawings

FIG. 1 depicts dose response curves for the viability of wild type and p53-deficient cells after irradiation.

FIG. 2 depicts dose response curves for the viability of wild type and p53-deficient cells after treatment with chemotherapeutic agents.

FIG. 3 depicts dose response curves for the viability of cells treated with sodium azide.

FIG. 4 depicts a 24-well plate for assaying putative anticancer agents at different concentrations in wild type and p53-deficient cells.

FIG. 5 depicts hypothetical dose response curves for the viability of wild type and p53-deficient cells after treatment with putative anticancer agents.

FIG. 6 depicts hypothetical dose response curves for plating efficiency of wild type and p53-deficient cells after treatment with putative anticancer agents.

FIG. 7 depicts hypothetical dose response curves for plating efficiency of wild type and p53-deficient cells after treatment with p53-dependent drugs and putative anticancer agents.

FIG. 8 depicts in vivo tumor response as a function of time to radiation or chemotherapy in mice injected with wild type or p53-deficient cells.

FIG. 9 depicts acquired resistance to radiation or chemotherapy of tumors derived from p53-expressing cells.

nonhuman animals. The term transgenic animal is meant to include an animal that gains new genetic information from the introduction of foreign DNA or a lesion, e.g., an in vitro induced mutation, e.g., a deletion or other chromosomal rearrangement, into the DNA of its cells. The animal may include a transgene in all of its cells including germ line cells, or in only some of its cells. Preferably, the transgenic animal is a mouse.

The term condition means a state of the cell. Such a condition includes, e.g., the state of the genome, the level of a gene product, and the introduction of any type of agent into or onto the cell. The state of the genome includes, e.g., a mutation in a gene and the addition of an exogenous gene. An agent includes, e.g., a molecule and radiation.

By apoptosis is meant programmed cell death. Such programmed cell death is at least in part a genetically controlled program essential for normal development and maintenance of tissue homeostasis. Cells undergoing apoptosis generally display one or more of shrinkage, loss of cell-cell contact, chromatin condensation and internucleosomal degradation of DNA. Many toxic stimuli induce apoptosis, even at doses or concentrations insufficient to cause general metabolic dysfunction. In addition, the expression of oncogenes may sensitize cells to apoptosis.

A condition which can reduce the susceptibility of a cell to apoptosis can include, e.g., a mutation in a gene that affects an apoptotic pathway. The term mutation is meant to include lesions which increase or decrease the level of expression of a gene affected by the mutation. The mutation can be homozygous or heterozygous. Genes that affect the apoptotic pathway include tumor suppressor genes, preferably the p53 gene, (Levine et al. Nature 351: 453-456, 1991), and regulators of tumor suppressor genes, e.g., the mdm2 gene which is a regulator for the p53 gene. Thus, absence of the p53 gene product reduces the susceptibility of

p53 is normally required for programmed cell death to occur, (ii) an expressed adenovirus E1A oncogene which in a wild type background enhances the susceptibility of the cell to apoptosis, and therefore results in cell death, but which in the absence of p53 gene product transforms the cell to a malignant state, and (iii) an expressed T24 H-ras oncogene which allows establishment of a permanent tumorigenic cell line when the expressing E1A oncogene is present.

Such a malignant cell is not killed by the cell's programmed cell death pathway (apoptosis) because of the absence of the apoptotic pathway p53 gene product. Treatments which are identified as being able to result in death of such cells are anticancer agents of great importance, in that p53 mutations are observed in many different types of cancers that are resistant or that become resistant to known chemotherapeutic drugs and ionizing radiation (Stretch et al., Cancer Res. 51: 5976-5979; Chiba et al., Oncogene 5: 1603-1610, 1990; Sidransky et al., Nature 355: 846-847, 1992; Felix et al., J. Clin. Invest. 89: 640-647, 1992; Yeargin et al., J. Clin. Invest. 91: 211-217, 1993.

The term treatment is meant to include an anticancer agent or putative anticancer agent that is administered. An anticancer agent can be, e.g., radiation or a molecule, e.g., a chemotherapeutic drug. Anticancer agents include, e.g., agents which kill cells, or prevent or retard growth and/or reproduction of cells. Preferably, the agents have a greater effect on actively dividing cells as compared to nondividing cells. Administration of the treatment includes introduction into, onto, in the vicinity of, e.g., into a medium in which the cell is suspended, or distal to, the cell. Depending upon the type of treatment, and the state of the cell, e.g., whether it is in a cell culture or part of an animal, the appropriate method for administration is chosen. The time, temperature, concentration and dose of the treatment depends

an anticancer agent by the methods of this invention described above, to alleviate the symptoms of cancer in an organism, is also provided.

One of the major problems with current cancer therapies is the appearance of resistant cell populations. This invention includes the administration of apoptotic pathway-dependent and apoptotic pathway-independent treatments, in combination, in order to minimize the problem of drug resistance. This problem is reduced by combination therapy because it is highly unlikely that the same cell would undergo mutations in both of these separate pathways. Therapeutic agents of the invention can be administered in any appropriate mode, e.g., orally, intravenously, subcutaneously, intraperitoneally, topically, combined with a liposome, in a time release formulation, transgenically, or by irradiation.

In a variation, the method provides for evaluating the ability of a treatment to bypass or restore or replace the function of a mutated or inactivated gene in the apoptotic pathway, e.g., a mutation in the tumor suppressor p53 gene. The method is similar to that described above, except that a treatment, e.g., a chemotherapeutic agent or radiation, that, preferably is known to kill the cell in the presence of tumor suppressor gene p53 activity, is administered in addition to administering the agent that is to be evaluated. In the absence of a functioning apoptotic pathway gene, the cell is not killed by the known chemotherapeutic agent or radiation. If the test agent restores or bypasses or replaces the function of the mutated or inactivated gene in the apoptotic pathway, the cell then becomes subject to programmed cell death.

This invention further provides a method for treating an organism, cell culture or cell, characterized by unwanted cell proliferation. Unwanted cell proliferation includes, e.g., cancer. By treating unwanted cell proliferation, e.g., cancer, it is meant alleviating the adverse effects of

protein or DNA. In the presence of the p53 protein or DNA, p53 deficient cells can become responsive to this sequence treatment.

This invention also includes mouse embryonic fibroblast cells which are wild type for the tumor suppressor gene p53 or are either homozygous or heterozygous for a tumor suppressor gene p53 mutation, and preferably also have an adenovirus E1A gene, and most preferably also have a T24 H-ras gene. This invention further includes mouse embryonic fibroblast cell lines comprising cells that are wild type for the tumor suppressor gene p53 or are either homozygous or heterozygous for a tumor suppressor gene p53 lesion and preferably also have an adenovirus E1A gene, and most preferably also have a T24 H-ras gene.

Deposit of MEF cells which $p53^{-/-}$ and have an adenovirus E1A gene and a T24 H-ras gene (strain 1AR.A9) has been made on August 19, 1993, with the American Type Tissue Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, and has been assigned deposit number CRL 11437. Deposit of MEF cells which are $p53^{+/+}$ and have an adenovirus E1A gene and a T24 H-ras gene (strain 1AR.C8) has been made on August 19, 1993, with the American Type Tissue Collection, 12301 Parklawn Drive, Rockville, MD 20852, and has been assigned deposit number CRL 11438.

EXAMPLES

Example 1 - Preparation of Embryonic Fibroblasts

$p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ mouse embryonic fibroblasts (MEFs) were obtained from 12-15 day embryos derived from crosses between mice in which one p53 allele had been disrupted by gene targeting. (Livingstone et al., Cell 70: 923-935, 1992). Mice with these genotypes are available from GenPharm International, Mountain View, California (TSG-p53 ® transgeneic mouse, item numbers P53101, P53102), P53201, P53202, P53301, P53302), and Jackson Laboratory, Bar Harbor, Maine (catalog # JR2080). The lack of p53 expression

Specifically, E1A was introduced into MEFs using p1AHygro, a plasmid derived by insertion of an Ad5 genomic E1A fragment (nucleotides 1-1834) (Lowe and Ruley, Genes Dev. 7: 535-545, 1993) into pY3 (a plasmid expressing hygromycin phosphotransferase) (Blochinger and Diggelmann, Mol. Cell. Biol. 4: 2929-2931, 1984). MEFs were seeded at 10^6 cells/100mm dish and allowed to adhere overnight. Exogenous DNA was introduced by calcium phosphate co-precipitation (Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, N.Y., 1989) using 1 μg p1AHygro to generate E1A containing cells. To generate E1A and T24 H-ras containing cells, 1μg p1AHygro plus a 10-fold molar excess of pT24 neo was used. pT24neo encodes an oncogenically activated human ras allele (T24 H-ras) expressed by its endogenous promoter. (Franza et al., Cell 44: 409-418, 1986). To generate E1A and E1B containing cells, 1 μg p1AHygro plus a 10-fold molar excess of p5XX was used. P5XX encodes the E1B gene. (Lowe and Ruley, Genes Dev. 7: 535-545, 1993). For each precipitation, the total mass of DNA was adjusted to 20 μg using a control plasmid, pBluescript, obtained from Stratagene of LaJolla, California. Precipitates were incubated with cells for 4 hours in the presence of 100 μM chloroquine, washed, and incubated for 24 hours in normal growth media. Subsequently, cultures were split into 3-5 150 mm plates and put on media containing either 100 μg/ml (for $p53^{-/-}$ MEFs) or 20 μg/ml (for $p53^{+/+}$ and $p53^{+/-}$ MEFs) Hygromycin B (obtained from Sigma Chemical Co. of St. Louis, Missouri). Fresh selection media was added every 3-4 days. After 2-3 weeks, colonies were either analyzed directly or expanded into permanent cell lines. All experiments were performed in hygromycin-free media.

This procedure results in oncogenes which enhance susceptibility to apoptosis in a wild type background being present in the cells at a level of at least about one copy per cell.

containing normal growth medium. Cell viability was assessed 36 hours after irradiation by pooling adherent and nonadherent cells and measuring uptake of fluoresceine isothiocyanate (FITC) by FACS analysis (Shi et al., J. Immunol. 144: 3326-3333, 1990; Lowe et al., Genes Dev. 7: 535-545, 1993). At least 10^5 cells were measured for each determination. Cells used in chemical cytotoxicity experiments were plated at 1-2 x 10^6 cells/100mm dish, allowed to adhere, and incubated with various concentrations of 5-fluorouracil, etoposide, adriamycin, or sodium azide (Sigma). Cell viability was determined 24 hours following treatment.

In order to confirm that cell death occurred by apoptosis, cells were analyzed for fragmented DNA. 24 hours after treatment, adherent and nonadherent cells were pooled and counted. Low molecular weight DNA was isolated from 2 x 10^6 cells and visualized following electrophoresis on 1% agarose gels and staining with ethidium bromide. Example 8 below sets forth the results obtained when following the above protocol.

Example 5 - Cell Cycle Analysis

Cellular proliferation was assessed by DNA content and incorporation of 5-bromo-2'-deoxyuridine (BrdU) using multiparameter flow cytometry analysis. Cells from Example 2 were plated in growth medium at 1-2 x 10^6 cells/100mm dish, allowed to adhere, and exposed to 5 Gy ionizing radiation. 14 hours after treatment, BrdU (obtained from Amersham Life Sciences of Arlington Heights, Illinois) was added and the cultures were incubated at 37°C for an additional 4 hours. Cultures were washed twice with PBS to remove dead cells, and the adherent cells were collected after treatment with trypsin. The cells were washed in PBS (phosphate buffered saline) and fixed in 70% ethanol for 30 minutes at -20°C. Samples were prepared and analyzed for multiparameter FACS analysis (van Erp et al., Cytometry 9: 627-630, 1988). The

TABLE 1

COLONY REGRESSION FOLLOWING TREATMENT WITH IRRADIATION

Treatment	Exogenous Genes	p53 Genotype	Colony Viability Regressing	Resistant	% Resistant
Radiation	E1A	(+/+)	5	1	17
	E1A	(+/-)	25	0	0
	E1A	(-/-)	1	24	96
	E1A + E1B	(+/+)	4	9	69
	E1A + E1B	(+/-)	9	16	64
	E1A + E1B	(-/-)	ND	ND	ND

While irradiation of untransfected fibroblasts had no effect on cellular viability, virtually all $p53^{+/+}$ and $p53^{+/-}$ colonies expressing E1A rapidly degenerated. By contrast, $p53^{-/-}$ colonies expressing E1A were resistant to irradiation, indicating that cell death required p53 function. Although not as effective as the absence of p53, co-expression of E1B protected E1A-expressing $p53^{+/+}$ cells from death following irradiation.

Example 7 - Genotoxic Compounds Used in Cancer Chemotherapy Induce p53-dependent Cell Death in Cells Expressing E1A in the Presence of the p53 Gene Product

A variety of genotoxic compounds used as chemotherapeutic agents were tested for their ability to induce p53-dependent cell death in E1A-expressing cells using the colony regression assay described in Example 3. 5-fluorouracil (anti-metabolite), etoposide (topoisomerase II inhibitor) and adriamycin (intercalating agent) were chosen because they have different intercellular targets (Chabner and Myers, in Cancer Principles and Practice of Oncology, DeVita et al., eds., J.B. Lippencott Co., Philadelphia, pp. 349-395, 1989).

p1AHygro was transfected into $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ MEFs obtained from Example 1 and colonies were selected in hygromycin B. Approximately 3 weeks after transfection, the colonies were marked and the liquid media

reaction assay that distinguishes between mutant and wild type p53 alleles, three out of four resistant colonies were shown to have lost the wild type p53 allele, and therefore had become deficient for p53. These data indicate that E1A increased cellular sensitivity to a number of chemotherapeutic agents and that ensuing cell death was dependent on a functional p53 gene.

Example 8 - Anticancer Agents Trigger p53-dependent Apoptosis in Cells Co-expressing E1A And T24 H-ras In The Presence Of The p53 Gene Product

The effects of various anticancer agents on permanent cell lines expressing E1A were examined. Although primary cells expressing E1A and endogenous p53 could not be expanded, $p53^{+/+}$ cells transformed by E1A and ras oncogenes (T24 H-ras), as described in Example 2, could be readily established. Cells expressing T24 H-ras and E1A remained susceptible to apoptosis upon serum withdrawal.

The viability of wild type and p53-deficient cells after irradiation was determined, as shown in FIG. 1. The viability of cells expressing (FIG. 1, panel A) or lacking (FIG. 1, panel B) endogenous p53 was measured by FITC uptake and FACS analysis 36 hours after treatment with the indicated dose of ionizing radiation. Each point represent the average and standard deviation obtained from at least 3 independent clones. All values were normalized to the relative viability of the corresponding untreated controls from the same experiment (generally greater than 90%). Open circles represent untransfected MEFs; closed circles, $p53^{-/-}$ clones expressing E1A; squares, clones co-expressing E1A and T24 H-ras; triangles, cells expressing E1A and E1B.

Like cells expressing E1A alone, exposure of $p53^{+/+}$ cells co-expressing E1A and T24 H-ras to ionizing radiation caused a dose-dependent decrease in viability, with significant death occurring at doses as low as 1 Gy.

co-expressing E1A and T24 H-ras were greater than 20-fold in this assay. No decrease in viability was observed in the untransfected MEFs of either p53 genotype following exposure to 5-fluorouracil and etoposide, even at doses as high as 100 μM 5-fluorouracil. However, the viability of $p53^{-/-}$ cells co-expressing E1A and T24 H-ras began to decline at higher concentrations of these two drugs, and the viability of all cells declined with increasing concentrations of adriamycin. Thus, at sufficiently high concentrations, these agents can cause cell death and have chemotherapeutic value in a p53-independent manner.

During apoptosis, loss of membrane integrity is typically preceeded by chromatin condensation and internucleosomal cleavage of genomic DNA (Wyllie, Nature 284: 555-556, 1980). As visualized by staining with 2,4-diamidino-2-phenylindole (DAPI), significant numbers of $p53^{+/+}$ cells co-expressing E1A and T24 H-ras contained condensed chromatin and fragmented nuclei within 8 hours of treatment with the various anticancer agents. In contrast, $p53^{-/-}$ populations rarely contained cells with altered chromatin structure. Low molecular weight DNA from cells exposed to ionizing radiation or chemotherapy treatment was analyzed.

Low molecular weight DNA was isolated from 2×10^6 cells 24 hours after exposure to ionizing radiation or treatment with chemotherapeutic agents. Cells were irradiated with 5 Gy (+) or incubated in media containing 1 μM 5-fluorouracil (5-FU), 0.2 μM etoposide (ETOP), or 0.1 μg/ml adriamycin (ADR). DNA was resolved by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining.

After irradiation, $p53^{+/+}$ cells co-expressing E1A and T24 H-ras contained large amounts of low molecular weight DNA, which produced a characteristic "ladder" on agarose gels. The degraded DNA was present in oligomers that were multiples of approximately 180-200 base pairs, suggesting

Example 10 - E1A Expression Allows Cells To Bypass p53-dependent Growth Arrest Following Irradiation

The effects of E1A and p53 expression on cell cycle progression following exposure to radiation and chemotherapeutic agents was determined. Cell cycle progression was assessed in cells exposed to ionizing radiation or 5-fluorouracil by 5-bromo-2'-deoxyuridine (BrdU) incorporation and measurement of cellular DNA content. $p53^{+/+}$ and $p53^{-/-}$ MEFs and E1A-expressing derivatives were incubated with BrdU for 4 hours beginning 14 hours after treatment. This represents a period when growth of irradiated fibroblasts is maximally inhibited and when $p53^{+/+}$ cells expressing E1A have initiated apoptosis. The relative amounts of cells in each phase of the cell cycle were estimated from the overall DNA content and the percentage of cells synthesizing DNA during the 4 hour BrdU pulse, as summarized in Table 3.

TABLE 3

CELL CYCLE PROGRESSION FOLLOWING EXPOSURE TO IONIZING RADIATION

Treatment	Cells	p53 Genotype	% OF TOTAL G0/G1	S	G2/M	%BrdU
none	MEF	+/+	61+/-3	17+/-4	22+/-5	28+/-4
	E1A + ras	+/+	34+/-3	47+/-3	19+/-1	74+/-1
	MEF	-/-	40+/-5	21+/-9	39+/-4	46+/-13
	E1A	-/-	27+/-1	52+/-2	21+/-2	79+/-1
	E1A + ras	-/-	23+/-2	62+/-2	15+/-0	89+/-2
5Gy	MEF	+/+	70+/-1	4+/-2	27+/-1	6+/-2
	E1A + ras	+/+	22+/-2	16+/-3	62+/-4	45+/-3
	MEF	-/-	35+/-3	18+/-5	47+/-2	43+/-13
	E1A	-/-	15+/-2	22+/-1	63+/-2	70+/-5
	E1A + ras	-/-	19+/-6	33+/-6	48+/-10	57+/-4

Untransfected MEFs and various clones were treated with 5 Gy ionizing radiation and incubated with BrdU. Cell proliferation was assessed by DNA content (propidium iodide staining) and incorporation of BrdU (using a FITC-anti-BrdU

disrupted gene's activity. Cytotoxicity of the agent between the oncogenically transformed cell lines and the parental MEFs (analogous to "normal," i.e., wild type cells) are compared. These assays identify agents that require p53, act independently of p53, or bypass p53 function.

The cytotoxicity of various putative anticancer agents is tested in cells transformed by E1A and T24 H-ras derived from either p53$^{+/+}$ or p53$^{-/-}$ MEFs. To determine whether an agent acts through p53, dose-response curves are performed on cells and cell viability is determined. Untransfected (early passage) MEFs expressing or lacking p53 are used as controls. Any one or combination of four assays is used as described below: a multiwell plate viability assay, a colony regression assay, a plating assay, and a progression or regression assay for tumors in animals.

A. Multiwell Plate Viability Assay

This assay is the easiest of the four assays and preferably is used for large screens. The assay uses 24-well plates and allows testing of one drug at five different concentrations, as shown in FIG. 4. Cells obtained from Examples 1 and 2 are plated into each well at a density of 2-4 x 10^3 cells/well and allowed to adhere. The next day, cells are treated with various concentrations of a drug, and analyzed for viability after approximately 24 hours. Dose response curves are generated plotting cell viability vs. drug concentration. Cell viability is determined by standard methods using a colorimetric assay, or vital dye exclusion or chromium release. (Clarke et al., Nature 362: 849-852, 1993; Vanhaesebroeck et al., Oncogene 8: 1075-1081, 1993; Duerkson-Hughes et al., J. Immunol. 143: 4193-4200, 1993). Values are normalized to the viability of untreated cells. The drug 5-fluorouracil (which kills by a p53-dependent mode) is included as a control.

Hypothetical results are shown in FIG. 5. The closed circles represent untransfected p53$^{+/+}$ MEFs; open circles, untransfected p53$^{-/-}$ MEFs; closed squares, p53$^{+/+}$ cells

Drugs that inhibit the reproductive potential of these cells reduce their ability to form colonies when plated at clonal density.

$p53^{+/+}$ and $p53^{-/-}$ cells transformed by E1A and T24 H-ras are plated at clonal density, approximately 10^3 cells/100 mm plate, in medium containing various concentrations of the agent to be tested. Medium is changed every 3-4 days until the colonies are large enough to be observed without magnification. Plates are fixed in formaldehyde and stained with crystal violet to assist in counting colonies. Values are normalized to the plating efficiency of untreated cells from the same cell line (# of colonies in drug/# colonies untreated). Drugs with known modes of action are used as controls.

Hypothetical results are shown in FIG. 6. Depending upon the pattern of results obtained for any given putative drug that is tested, a determination of its anticancer properties are made. The closed squares represent $p53^{+/+}$ cells co-expressing E1A and T24 H-ras; open squares, $p53^{-/-}$ cells co-expressing E1A and T24 H-ras. In panel A, a p53-dependent mode of killing (or inhibition of reproductive potential) is shown, and in panel B, a p53-independent mode is shown.

D. Progression or Regression Assay of Tumors in Animals

Cells transformed by E1A and T24 H-ras are highly tumorigenic. Therefore, these cells can be used to generate tumors in animals for tests of drug effectiveness *in vivo* as well as initial estimates of drug toxicity in the animal. Since the MEFs used to generate the cell lines differ only in the status of the disrupted apoptotic gene, differences in tumor response to therapy are attributable to the disrupted gene.

For p53, cellular sensitivity to p53-dependent apoptosis in cells expressing E1A and ras is not selected against during tumor development in nude mice, suggesting that tumors can be generated that are either sensitive or resistant to

ii. Identifying Drugs that Do Not Require p53 Function for Cytoxicity

Not all forms of apoptosis require p53 function, (Clark et al., Nature 362: 849-852, 1993), suggesting that potentially useful drugs may activate apoptosis independently of p53 or "downstream" of p53 in the p53-dependent apoptotic pathway. Such a drug kills $p53^{+/+}$ and $p53^{-/-}$ cells expressing E1A and ras equally (see FIG. 5, panels B and C; FIG. 6, panel B). Moreover, this drug should have minimal toxicity with respect to untransfected fibroblasts (multiwell plate viability assay). Similarly, a p53-independent drug induces regression of both $p53^{+/+}$ and $p53^{-/-}$ tumors, with minimal toxicity at the active concentration (progression or regression assay of tumors in animals).

iii. Identifying Molecules that Bypass, Restore or Replace p53 Function

Anticancer agents that act by restoring, bypassing or replacing p53 activity can be identified by the methods of the invention. Hypothetical results using the plating assay are shown in FIG. 7. Depending upon the pattern of results obtained for any given putative drug that is tested, a determination of its anticancer properties is made. The closed squares represent $p53^{+/+}$ cells co-expressing E1A and T24 H-ras; open squares, $p53^{-/-}$ cells co-expressing E1A and T24 H-ras. Solid lines represent a p53-dependent drug (e.g. 5-fluorouracil) alone; dashed lines, a p53-dependent drug plus the drug that bypasses or restores p53 function. When the p53-independent drug is used in combination with a p53-dependent drug (e.g., 5-fluorouracil), the concentration of the 5-fluorouracil required to kill 50% of the p53-deficient cells expressing E1A and ras is reduced.

Such p53 drugs may also be tested using the progression or regression assay of tumors in animals to determine their usefulness in vivo.

cells are subjected to multiple rounds of infection at high multiplicity of infection (i.e., # of infectious units/cell), in order to transfect as many cells as possible. Incubation of cultured cells in the presence of polybrene during infection increases the infection frequency.

Alternatively, introduction of wild type p53 DNA into the p53-deficient cells is accomplished by gene transfer of p53-expressing plasmids. (Shaw et al., Proc. Nat'l. Acad. Sci. U.S.A. 89: 4495-4499, 1992; Baker et al., Science 249: 912-915, 1990).

The transfected cells are subjected to treatment with p53-dependent anticancer agents, or a combination of p53-dependent and p53-independent anticancer agents. Since the transfected cells contain a wild type p53 gene, a p53-dependent response to the anticancer agents is effected, e.g., a retardation of unwanted cell growth and/or reproduction, destruction of cells, or a loss of viability or unwanted characteristics.

Example 14 - Introduction of Wild Type p53 DNA into Mice with Tumors Lacking p53

Mice lacking tumor suppressor gene p53 have been generated (Donehower et al., Nature 356: 215-220, 1992; Clarke et al., Nature 362: 849-852, 1993), and are available as described in Example 1. Mice homozygous for p53-deficiency develop spontaneous tumors primarily of thymic origin, i.e., T cell lymphoma (Donehower et al., Nature 356: 215-220, 1992). Furthermore, mice heterozygous for p53-deficiency develop a wide range of tumors (at later times than the homozygous mice), which are similar to humans with Li-Fraumeni syndrome (Malkin et al., N. Engl. J. Med. 326: 1309-1315, 1992), and therefore provide a good model for human cancer. In tumors that develop from heterozygous mice, the wild type p53 gene is frequently lost or mutated.

These mice, or other "knock-out" mice with mutations in an apoptotic pathway, provide a useful system for determining

Example 16 - In Vivo Tumor Response to Anti-Cancer Therapies Depends Upon the Presence of p53

This example illustrates that the presence of p53 has a dramatic effect on tumor response to γ-irradiation and chemotherapy in vivo.

A transplantable fibrosarcoma model was developed to investigate the effects of p53 on tumor growth and sensitivity to drug and radiation therapy in vivo. Embryonic fibroblasts derived from wild type mice and mice which lack functional p53 genes were oncogenically transformed by the combination of adenovirus early region 1A (E1A) and an activated ras oncogene (T24-H ras). These cells can form tumors following subcutaneous injection into nude mice regardless of their p53 status, but cells which express p53 remain sensitive to apoptosis in vitro. Lowe et al., Proc. Natl. Acad. Sci. U.S.A. 91:2026 (1994); Lowe et al., Cell 74: 957-967 (1993). This oncogene combination provides a well-characterized system of multi-step carcinogenesis (Ruley, Nature 304: 602 (1983)), analogous to many naturally occurring tumors. Since these cells are highly oncogenic, tumor growth can occur without strong selection for additional mutations. It was therefore possible to compare tumors that differed primarily in their p53 status.

Oncogenically transformed cells expressing p53 formed fewer tumors, and with a longer latency, than p53-deficient cells. These results were in agreement with earlier studies. Lowe et al., Proc. Natl. Acad. Sci. U.S.A. 91:2026 (1994). Cells lacking p53 gave rise to tumors at all injected sites compared to 82 ± 24% ($p<0.11$) of sites injected with p53-expressing cells, with an average latency of 8 ± 4 and 18 ± 7 days, respectively ($p<0.03$). However, after reaching a palpable size, tumors derived from both wild type and p53-deficient cells expanded at similar rates (Fig. 8), until the tumors became sufficiently large (>1 cm^3) that the animals were sacrificed.

TABLE 4

SUMMARY OF TUMOR RESPONSE TO RADIATION AND CHEMOTHERAPY

Clone	p53	Treatment	n	Tumor Response: Min.Vol.	Tumor Response: d	Tumor Response: Vol.(d7)
C1	(+/+)	7Gy	2	53	6	53
		9Gy	2	40	7	40
		12Gy	4	11±10	7	10
		Adr	2	42	3	NA
C6	(+/+)	7Gy	2	50	3	89
		9Gy	3	42±27	4	67
		12Gy	2	20	4	37
		Adr	2	13	6	NA
C8	(+/+)	2Gy	3	29±12	8	64
		5Gy	3	8±13	5	12
		7Gy	3	16±9	7	17
		Adr	6	19±13	6	15
C10	(+/+)	5Gy	2	96	1	133
		7Gy	2	82	5	117
		12Gy	2	89	3	95
		Adr	1	100	0	186
A4	(-/-)	7Gy	2	100	0	230
		12Gy	2	100	0	305
		Adr	4	100±0	0	289
A8	(-/-)	7Gy	4	91±13	0	177
		12Gy	2	100	0	125
A9	(-/-)	7Gy	2	95	1	223
		12Gy	4	100±0	0	171
E6	(-/-)	7Gy	2	100	0	177
		Adr	4	100±0	0	232

NA, not applicable

cells were even more sensitive to higher doses of radiation, p53-deficient tumors were refractory to levels as high as 12 Gy (Fig. 8, panels C and D, and Table 4).

p53 also influenced the effectiveness of chemotherapy. Specifically, adriamycin treatment induced a rapid regression of tumors derived from wild type cells but did not significantly affect the growth of p53-deficient tumors (Fig. 8, panels E and F). The amount of adriamycin used in these experiments approached the maximum tolerated dose for a single injection (Scheulen et al., Strahlentherapie Onkologie 165:529 (1989)). Thus, while higher doses of adriamycin are capable of killing p53-deficient cells in vitro (Lowe et al., Cell 74: 957 (1993), the concentrations required for this effect are toxic to the animal. The one tumor derived from p53-expressing cells that failed to respond to adriamycin was derived from the same clone that was relatively radioresistant (C10, Table 4).

Example 17 - In Vivo Regression of Tumors Derived From p53-Expressing Cells Subjected to Anti-Cancer Therapies Is Due to Apoptosis

This example illustrates that the regression of tumors derived from p53-expressing cells subjected to γ-irradiation or chemotherapy is due to apoptosis, and that p53-deficient tumors which do not regress are less susceptible to apoptosis.

In general, cells that undergo apoptosis are interspersed throughout tumors and have distinct morphological features, including cell shrinkage, chromatin condensation, and loss of extracellular contacts. Kerr et al., Br. J. Cancer 26: 239 (1972). Apoptotic cells often activate an endonuclease that breaks genomic DNA (Wyllie, Nature 284: 555 (1980)), generating products which are readily detected in situ using the terminal deoxytransferase-mediated dUTP-biotin nick end labeling (TUNEL) method. Gavrieli et al., J. Cell Biol. 119: 493 (1992).

treated with either γ-irradiation or adriamycin contained few regions of pyknosis and cell loss. Nevertheless, there was a small but reproducible increase in the number of TUNEL-positive cells interspersed throughout the tumor. These data indicate that the regression of tumors derived from p53-expressing cells was due to apoptosis, and that p53-deficient tumors, which did not regress were less susceptible to apoptosis.

Example 18 - Tumors That Become Resistant to Anti-Cancer Therapies In Vivo Can Result From a p53 Mutation

This example illustrates that initially responsive tumors derived from p53-expressing clones display a reduced response to subsequent treatments and several become completely resistant.

Moreover, tumors derived from one p53-expressing clone (C10) responded poorly to the initial treatments with radiation and chemotherapy (Fig. 9 and Tables 4 and 5), even though the injected cells readily underwent apoptosis *in vitro* (not shown).

Since an intact p53-dependent apoptotic program is essential for significant tumor regression, it was tested whether acquired resistance involved loss of p53 function by mutation. Regions of tumor cDNAs corresponding to exons 5-8 of the p53 gene were amplified by polymerase chain reaction (PCR) and sequenced. This region contains greater than 90% of the mutations responsible for p53 inactivation in human tumors. Hollstein et al., Science 253:49 (1991). Amplification and sequences were performed as follows. Tumor cells were dispersed with trypsin and cultured in medium containing 50 μg/ml hygromycin B to select for tumor-derived cells (which contain hygromycin phosphotransferase, Lowe et al., Proc. Natl. Acad. Sci. U.S.A. 91:2026 (1994)). RNA was obtained from the tumor cells maintained for less than about one week in culture. For tumor 915L, RNA was obtained directly from frozen tumor

The results are shown in Table 5. For each tumor, the treatment(s) are indicated as the dose of ionizing radiation (in Gy) or as adriamycin treatment (A). In parentheses is the minimum volume achieved following treatment, normalized to the tumor volume on the day of treatment. Also shown is the detected nucleotide change, the affected codon in murine p53 (Bienz et al., Embo J. 3:2179 (1984)), the corresponding human codon (Hollstein et al., Science 253:49 (1991)), and the predicted amino acid substitution.

As summarized in Table 5, p53 mutations were detected in approximately 50% of the resistant or relapsed tumors, at codons observed mutated in human cancer. Hollstein et al., Science 253:49 (1991). One untreated tumor and two nonresponsive tumors derived from clone C10 acquired a missense mutation (histidine to arginine) at codon 211. No wild type sequence was detected in these tumors, suggesting that they had lost the normal p53 allele. Although not detectable in the injected population, cells harboring this mutation presumably represented a small percentage of the injected cells and were selected during tumor growth. This probably accounts for the poor response of all tumors derived from clone C10.

In tumors arising from clone C6, p53 mutations were detected in recurrent tumors that were previously treated with high levels of ionizing radiation (Table 2). While no p53 mutations were detected in the injected cells (not shown), a phenylalanine to cystine substitution was observed at codon 131 in each relapse tumor analyzed, again suggesting that a small percentage of parental cells harbored this mutation. A second missense mutation (cystine to serine at codon 239) was observed in only one C6-derived tumor (#977R), implying it was acquired during tumor expansion.

Fig. 9 shows the acquired resistance of tumors derived from p53-expressing clones. Athymic nude mice were injected with transformed embryonic fibroblasts (time 0) and treated as described in Fig. 8. (A) Animals harboring tumors derived

CLAIMS

1. A method for evaluating the ability of a treatment to adversely affect the growth or viability of a cell, comprising:

providing a cell having a first condition which reduces the susceptibility of said cell to apoptosis;

administering said treatment to said cell; and

determining whether said treatment affects the value of a parameter related to the growth or viability of said cell.

2. The method of claim 1 wherein said cell is obtained from a cell culture.

3. The method of claim 1 wherein said cell is a mouse embryo fibroblast.

4. The method of claim 1 wherein said cell is part of an organism.

5. The method of claim 4 wherein said organism is an animal, said animal selected from the group consisting of an animal being homozygous for a mutation in the apoptotic pathway, an animal being heterozygous for a mutation in the apoptotic pathway, and an animal being wild type for the apoptotic pathway.

6. The method of claim 5 wherein said animal is a transgenic animal.

7. The method of claim 1 wherein said first condition includes a mutation in a gene that affects the apoptotic pathway.

8. The method of claim 7 wherein said mutation is homozygous.

20. The method of claim 16 wherein said cell further comprises a third condition which allows establishment of a permanent cell line when said second condition is present and comprises an expressed oncogene.

21. The method of claim 20 wherein said third condition allows said cell to form a tumorigenic cell line.

22. The method of claim 20 wherein said third condition comprises an expressed oncogene.

23. The method of claim 22 wherein said oncogene is T24 H-ras.

24. The method of claim 1 wherein said first condition is a mutation in the tumor suppressor gene p53, said second condition is an expressed adenovirus E1A gene, and said third condition is an expressed T24 H-ras gene.

25. The method of claim 1 further comprising administering a second treatment to said cell which in the presence of tumor suppressor gene p53 activity would reduce the growth or viability of said cell.

26. The method of claim 25 wherein said second treatment is selected from the group consisting of a chemotherapeutic agent and radiation.

27. A method for identifying an agent useful for treating unwanted cell proliferation, comprising:

providing a cell comprising a first condition which reduces susceptibility of said cell to apoptosis and a second condition which in a wild type background enhances susceptibility of said cell to apoptosis;

comparing the values of said growth or viability parameter between said first cell and said second cell such that if said treatment inhibits said parameter more extensively in said first cell than in said second cell, then the treatment is apoptotic pathway dependent and identifies the treatment as being effective for cells which are wild type for the apoptotic pathway.

33. The method of claim 32 wherein said mutation is in the tumor suppressor p53 gene.

34. The method of claim 32 wherein said mutation is homozygous.

35. A method of utilizing an anticancer drug to alleviate the symptoms of cancer in an organism, which anticancer drug has been originally selected as an anticancer agent by:

providing a cell having a first condition which reduces the susceptibility of said cell to apoptosis;

administering a first treatment to said cell; and

determining whether said first treatment affects the value of a parameter related to the growth or viability of said cell.

36. The method of claim 35 further comprising utilizing a second treatment selected from the group consisting of a chemotherapeutic agent and radiation to alleviate the symptoms of cancer in the organism.

37. A method of treating a cell which is tumorigenic, said cell being part of an organism, by administering tumor suppressor gene p53 DNA in an amount sufficient to give expression of a therapeutically effective amount of p53 protein.

45. A method of treating an organism with cancer, comprising:

testing said organism's cancer cells for the presence or absence of a tumor suppressor gene p53 mutation; and

if a p53 mutation is absent, then administering a therapeutically effective amount of a treatment selected from the group consisting of a p53-dependent treatment, and a combination of a p53-dependent and a p53-independent treatment; and

if a p53 mutation is present, then administering a therapeutically effective amount of a treatment selected from the group consisting of a p53-independent treatment, and a sufficiently high level of a normally p53-dependent treatment so as to overcome the p53-dependent property of said p53-dependent treatment.

46. The method of claim 45 wherein said screening is selected from the group consisting of immunohistochemical analysis of tumors using mutant-specific antibodies, single strand polymorphism analysis for detecting point mutations and loss of heterozygosity, nondenaturing gradient gel electrophoresis, direct sequencing, and RNase protection assays.

47. A mouse embryonic fibroblast cell, said cell being homozygous for a tumor suppressor gene p53 mutation and having an adenovirus E1A gene and a T24 H-ras gene.

48. A mouse embryonic fibroblast cell, said cell being heterozygous for a tumor suppressor gene p53 mutation and having an adenovirus E1A gene and a T24 H-ras gene.

49. A mouse embryonic fibroblast cell line comprising cells that are homozygous for a tumor suppressor gene p53 mutation and having an adenovirus E1A gene and a T24 H-ras gene.

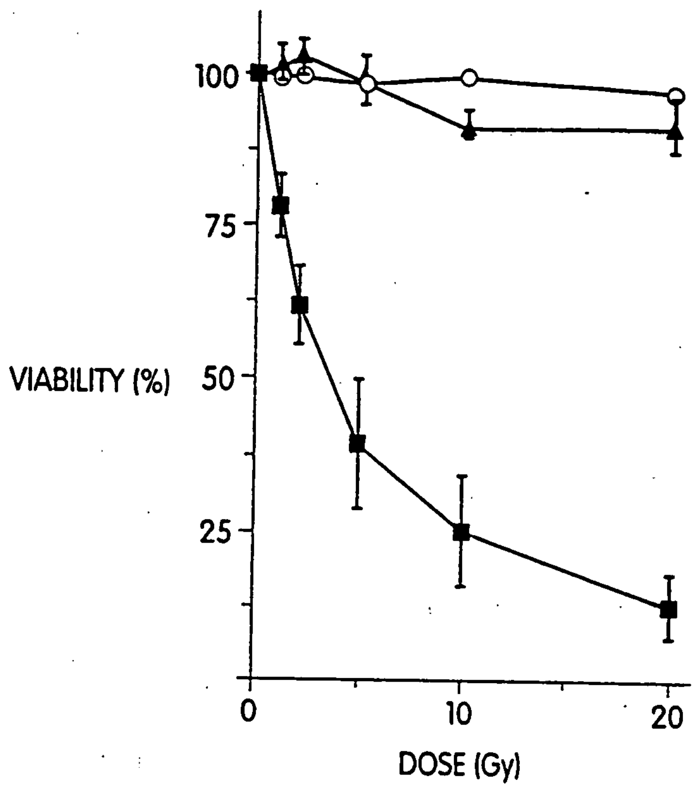
VIABILITY (%)
100
75
50
25
0
10
20
DOSE (Gy)

Fig. 1A

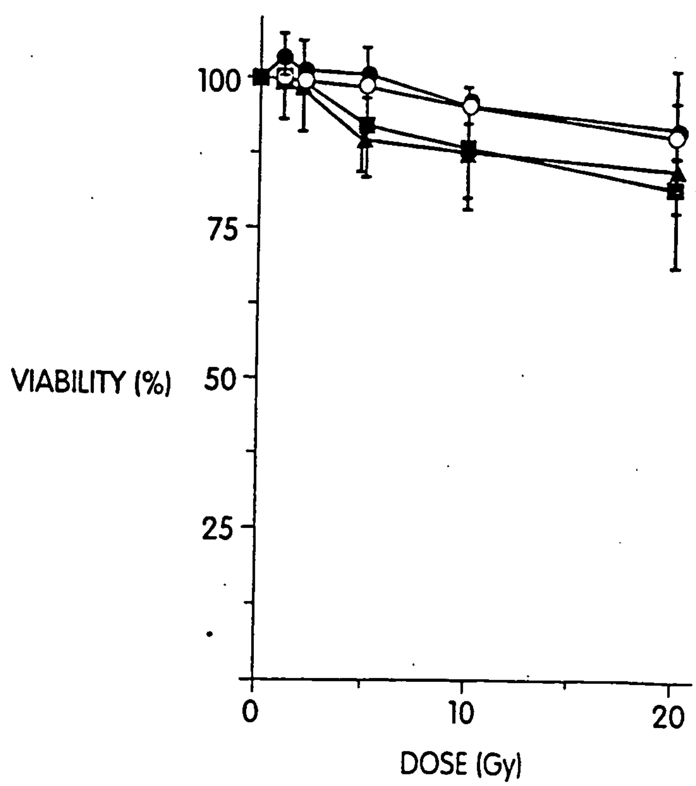
VIABILITY (%)
100
75
50
25
0
10
20
DOSE (Gy)

Fig. 1B

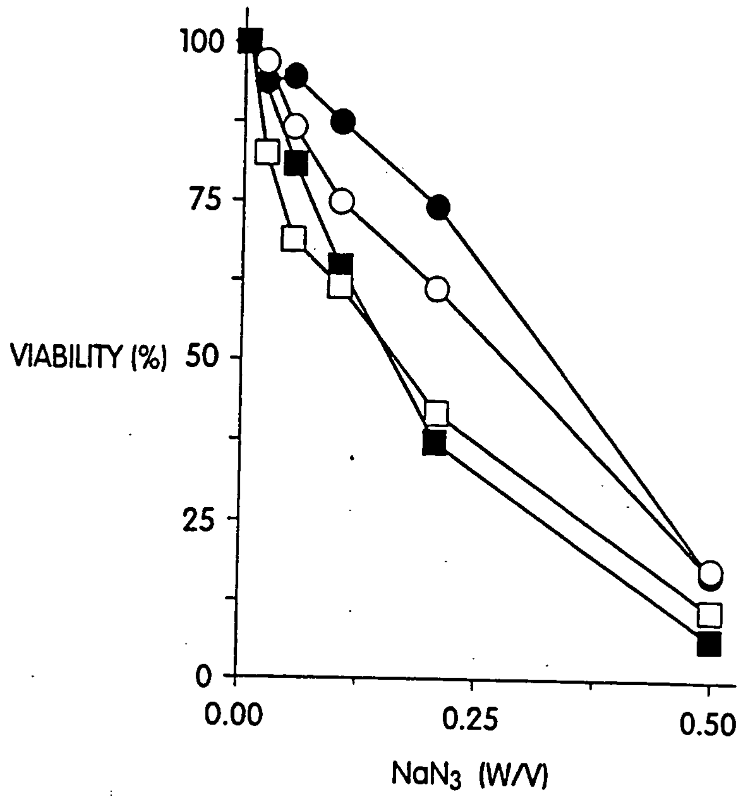
100
75
VIABILITY (%) 50
25
0
0.00
0.25
0.50
NaN_3 (W/V)

Fig. 3

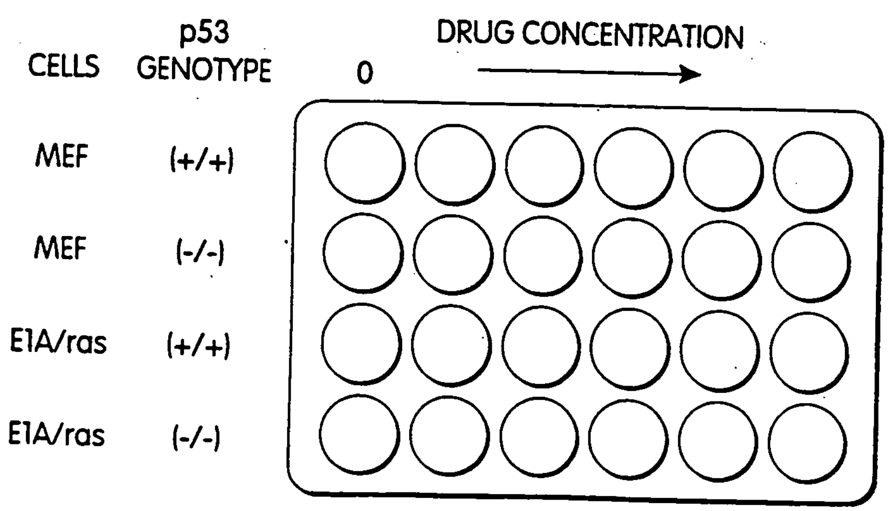
CELLS
p53 GENOTYPE
DRUG CONCENTRATION
0
MEF (+/+)
MEF (-/-)
E1A/ras (+/+)
E1A/ras (-/-)

Fig. 4

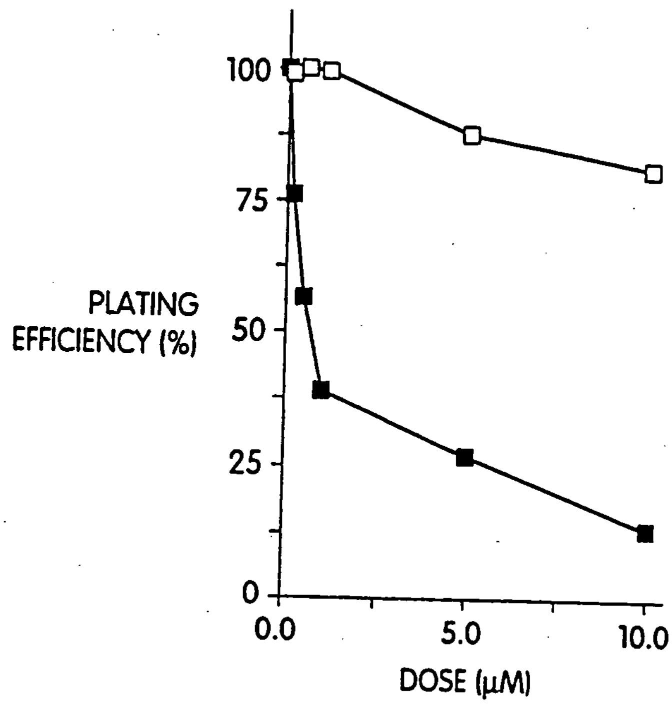
PLATING
EFFICIENCY (%)
100
75
50
25
0
0.0
5.0
10.0
DOSE (μM)

Fig. 6A

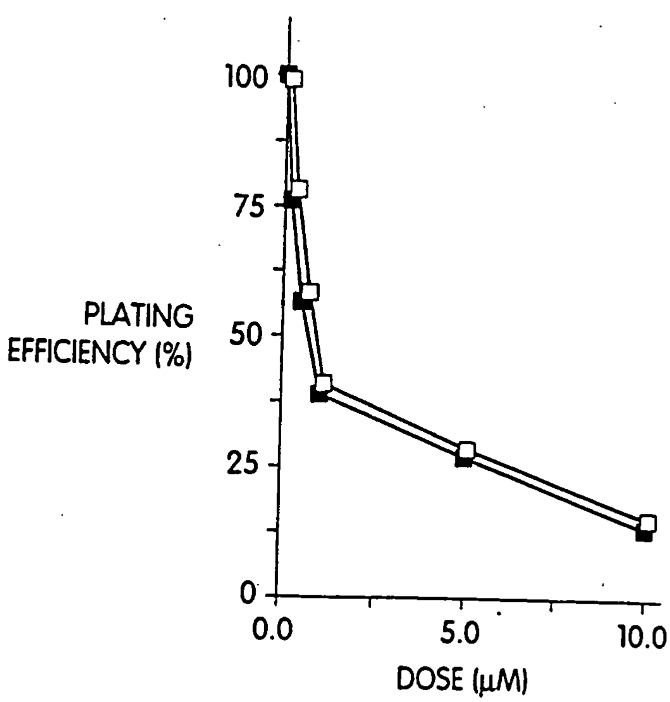
PLATING
EFFICIENCY (%)
100
75
50
25
0
0.0
5.0
10.0
DOSE (μM)

Fig. 6B

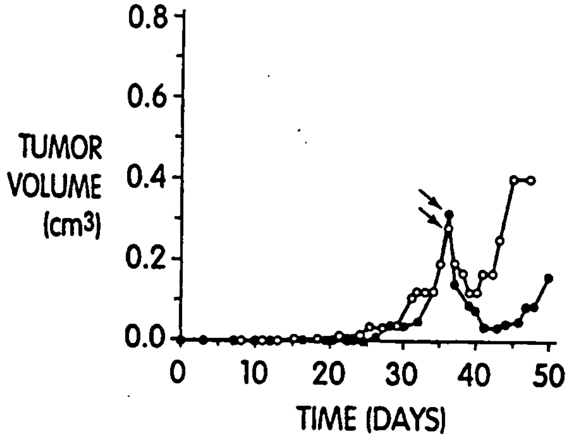

Fig. 8A

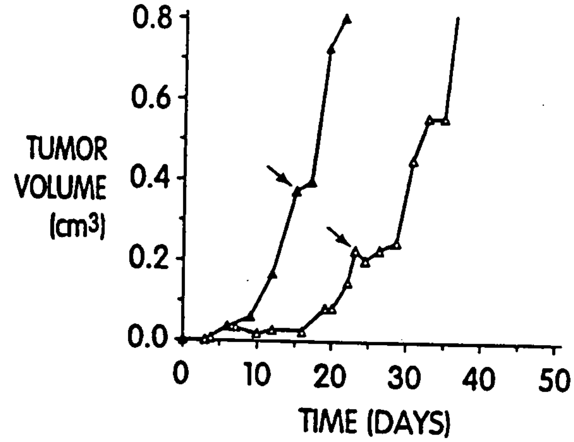

Fig. 8B

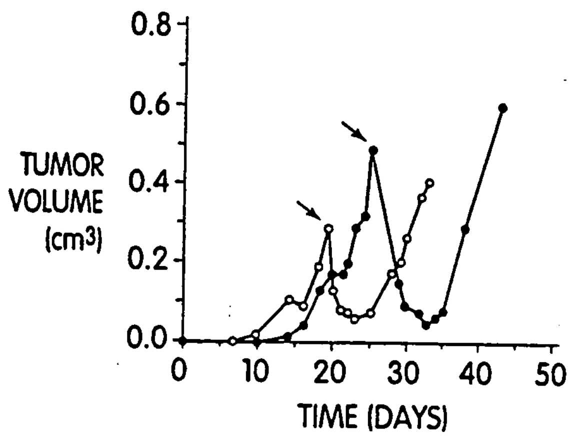

Fig. 8C

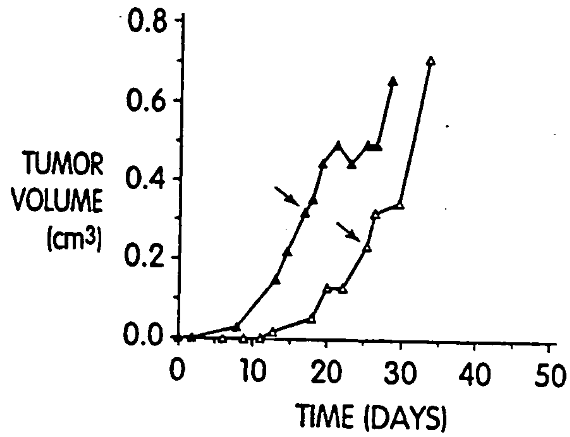

Fig. 8D

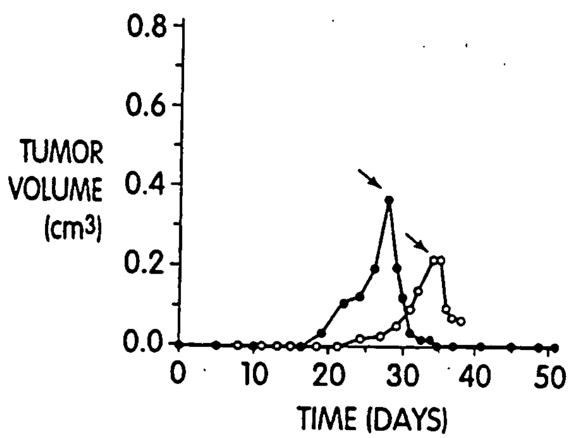

Fig. 8E

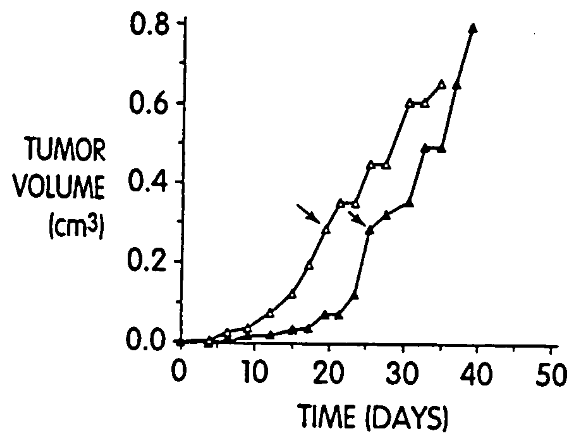

Fig. 8F

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 43/38; A61K 31/40

US CL :514/410

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/410

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN CAS file Medline

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BLOOD, Volume 82(1), issued 01 July 1993, Sachs L. et al., "Control of Programmed Cell Death in Normal and Leukemia Cells: New Implications for Therapy", pages 15-21, see Medline Abstract No.: 93313190 only.	1-50

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 NOVEMBER 1994	12 DEC 1994

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